Review

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Current status of genome editing in vector mosquitoes: A review

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Summary **Mosquitoes pose a major threat to human health as they spread many deadly diseases like malaria, dengue, chikungunya, filariasis, Japanese encephalitis and Zika. Identification and use of novel molecular tools are essential to combat the spread of vector borne diseases. Genome editing tools have been used for the precise alterations of the gene of interest for producing the desirable trait in mosquitoes. Deletion of functional genes or insertion of toxic genes in vector mosquitoes will produce either knock-out or knock-in mutants that will check the spread of vector-borne diseases. Presently, three types of genome editing tools viz., zinc finger nuclease (ZFN), transcription activator-like effector nucleases (TALEN) and clustered regulatory interspaced short palindromic repeats (CRISPR) and CRISPR associated protein 9 (Cas9) are widely used for the editing of the genomes of diverse organisms. These tools are also applied in vector mosquitoes to control the spread of vector-borne diseases. A few studies have been carried out on genome editing to control the diseases spread by vector mosquitoes and more studies need to be performed with the utilization of more recently invented tools like CRISPR/Cas9 to combat the spread of deadly diseases by vector mosquitoes. The high specificity and flexibility of CRISPR/Cas9 system may offer possibilities for novel genome editing for the control of important diseases spread by vector mosquitoes. In this review, we present the current status of genome editing research on vector mosquitoes and also discuss the future applications of vector mosquito genome editing to control the spread of vector-borne diseases.**

Keywords: Gene alteration, mosquito borne disease control, zinc finger nuclease (ZFN), transcription activator-like effector nuclease (TALEN), clustered regulatory interspaced short palindromic repeats (CRISPR)/CRISPR associated protein 9 (Cas9)

1. Introduction

Mosquitoes are arthropod vectors responsible for the transmission of several disease causing pathogens. Dengue, chikungunya, malaria, filariasis and Japanese encephalitis are the major mosquito borne diseases responsible for thousands of deaths each year (*1,2*). A recent outbreak of Zika is also caused by a vector mosquito *Aedes aegypti*. The World Health Organization declared a Public Health Emergency of International Concern due the outbreak of Zika in South America (*3*). So mosquitoes pose a great threat to public health and affect the economy of several countries. For the past several decades, synthetic insecticides were being used to control vector mosquitoes. Unfortunately, synthetic insecticides cause environmental pollution and kill many beneficial insects (*4,5*). Further, continuous use of synthetic insecticide has also resulted in the development of resistance in vector mosquitoes (*6*).

Recent advances in the field of genetic technologies

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have strengthened our understanding on creation of anti-pathogenic mosquito strains (*7-10)*, sterile mosquito strains (*11,12*) and genetically modified strains (*13-15*). Especially the discovery of modern genome editing technologies provide many opportunities to edit new target genes, to analyze the functions of target genes more accurately and to modify the expression levels of target genes (upregulation or downregulation). As per Criscione *et al*., 12 different classes of genetic-based technologies have been used as functional genomic tools for the control of insect vectors (*16*). Genome editing technology is one among them and it has been emerging as a powerful tool that can alter the genome more precisely.

Three types of genome editing tools are widely used for engineering the genomes of diverse species including vector mosquitoes. These are zinc finger nuclease (ZFN), transcription activator-like effector nucleases (TALEN) and clustered regulatory interspaced short palindromic repeats (CRISPR) and CRISPR associated protein 9 (Cas9). The ZFN based genome editing technique was initially applied in *Drosophila melanogaster* (*17,18*). This approach stimulated diverse ideas to carry out modifications in the genome of any insect. Following this, researchers have reported the successful application of ZFN and TALEN based genome editing technology in plants (*19-22*) and other animals (*23,24*). However after the discovery of CRISPR/Cas9 in 2012 (*25*), many researchers have successfully applied this technique to diverse organisms including mosquitoes and cell lines for precise genome editing. These genome editing technologies enable the alteration of target genes in insect pest, particularly useful for the control of vector-borne diseases caused by mosquitoes. Although excellent reviews are available on mosquito genome editing with these techniques (*26-32*), we present the recent application of these techniques in vector mosquito gene manipulation for the control mosquito borne diseases especially by CRISPR/Cas9, off-target effects of these tools, ethical issues and current problems in application of genome editing techniques in vector mosquitoes.

2. Major genome editing tools

Three major genome editing tools are currently applied for target specific alteration of genomes of diverse organisms. These are ZFN, TALEN and CRISPR/Cas9, although other techniques like meganuclease-mediated genome editing are proposed. ZFNs are one of the genome editing tools developed initially which was based on the specificity of DNA binding protein ZFN. ZFN is a target-specific endonuclease designed to bind and cleave DNA at desired positions of the genome. ZFN consists of DNA binding domain with zinc finger which recognizes the specific sequence on the genome and nuclease domain made up of *FolkI* enzyme which cleaves the specific site of DNA. DNA-binding domains of individual ZFNs typically contain three to six individual zinc finger repeats and each finger can recognize 3 base pairs. Through this strategy it is possible for ZFN to induce double-stranded breaks (DSB) at a specific region on the genome and with the help of endogenous DNA repair this technique was used by several groups to accurately alter the genome sequence of higher organisms (*33-36*). TALEN based genome editing is easy to engineer compared to ZFN and it is also more specific to target sequence (*37*). DNA binding domain of TALEN contains a highly conserved repeat of 33-34 amino acid sequence with difference at 12^{th} and 13^{th} amino acids. These two positions are highly variable and show a strong correlation with specific nucleotide recognition in the genome. This relationship between amino acid sequence and DNA recognition enabled the engineering of specific DNA-binding domains. DNA binding domain is fused with the *FokI* nuclease enzyme which confers extreme site specificity and has expanded the possibility of specific editing in a number of genomes (*38,39*).

CRISPR/Cas9 is a RNA-guided endonuclease technology that has been considered as a highly versatile tool for making breaks in the genomes of bacteria, yeast, plants and animals. CRISPR/Cas9 is the latest addition in the genome editing tool box. Compared to ZFN and TALEN, the creation of CRISPR/Cas9 constructs is several times easier and it is also more convenient to handle. CRISPR/Cas9 was found to function as an acquired immune system against viruses and phages through CRISPR RNA (crRNA)-guided DNA binding and Cas9 nucleases-mediated DNA breakage in bacteria and archaea (*40*). In genome editing, CRISPR/Cas9 works with the help of the single guide RNA (sgRNA) which recognises the target sequence (protospacer) in the genome of host organism through complementary base pairing (*25*). Then the Cas9 nuclease specifically makes a double-stranded break (DSB) at a region near to the PAM (Protospacer Adjacent Motif) sequence. The invention of sgRNAs was the major breakthrough in this field which was initially used along with Cas9 to create breaks in various DNA sites *in vitro* (*25*). Following this, several papers have been published utilizing this technology for precise genome engineering in cell lines and in diverse organisms (reviewed in *41-43*).

3. Application of genome editing in vector mosquitoes

The invention and rapid development of tools like CRISPR/Cas9 have significantly expanded the scope of genome editing research that can be achieved in a broad range of organisms including vector mosquitoes. Further, the well established procedures are additional advantages which increase our ability to work on manipulation of mosquito genome. As per Franz *et al*. genome editing

Table 1. Details on various genome editing studies undertaken in mosquitoes using ZFN, TALEN and CRIPR/Cas9 systems

Mosquito species	Genome editing tool	Targeted genes	Application	*Ref.*
Ae. aegypti	ZFN	*AaegGr3*	Disruption of sensory pathways-dengue control	2014 (*61*)
Ae. aegypti	ZFN	*Orco*	Disruption of odorant receptor pathways-dengue control	2013 (*14*)
Ae. aegypti	ZFN	*npylr1*	Disruption of blood feeding behavior-dengue control	2013 (*60*)
Ae. aegypti	TALEN	*Kmo*	Lack of eye pigmentation-dengue control	2013 (*13*)
An. gambiae	TALEN	*TEP1*	Immune pathways-malaria control	2013 (*15*)
Ae. aegypti	CRISPR/Cas9	*ECFP*	Functional genomics	2015 (*65*)
Ae. aegypti	CRISPR/Cas9	*Nix*	Conversion of females into harmless males	2015 (*66*)
Ae. aegypti	CRISPR/Cas9	*Aaeg-wtrw*	Site-specific mutations	2015 (*63*)
Ae. aegypti	TALEN	*dcr2 and ago2*	Transgenic strains and gene drive	2015 (*64*)
Ae. aegypti	CRISPR/Cas9	*Kmo, loqs, r2d2, ku70, lig4* and *nix genes*	Transgenic strains and gene drive	2015 (*64*)
An. stephensi	CRISPR/Cas9	*M1C3* and *m2A10*	*P. falciparum* resistance strains - malaria control	2015 (*67*)
An. gambiae	CRISPR/Cas9	*AGAP005958, AGAP007280* and *AGAP011377*	*An. gambiae* population suppression - malaria control	2016 (*68*)
An. gambiae	CRISPR/Cas9	*X-linked rDNA sequence*	Sex-distortion in *An. gambiae* - malaria control	2016 (*69*)

Abbreviations of genes: *AaegGr3*: *Ae. aegypti* gustatory receptors, *orco*: odorant receptor coreceptor, *npylr1*: neuropeptide Y-like receptors 1, *kmo*: kynurenine 3-monoxygenase, *TEP1*: thioester-containing protein, *ECFP*: enhanced cyan fluorescent protein, *Nix*: male-determining factor gene, *Aaeg-wtrw*: *Ae. aegypti* water witch locus, *dcr2*: dicer2, *ago2*: argonaute2, *loqs*: loquacious, *r2d2*: *r2d2* protein, *ku70*: ku heterodimer protein gene, *lig4*: ligase4, *m1C3* and *m2A10*: antiparasite effector genes, *AGAP005958*, *AGAP007280* and *AGAP011377*: *An. gambiae* female-fertility genes. In all these methods, the constructs were delivered through embryonic microinjection.

of vector mosquitoes is aimed for three major purposes: *i*) vector and pathogen control, *ii*) study of target gene function and *iii*) to improve genetic manipulation (*44*). We have summarized the successful reports of genome editing in vector mosquitoes in Table 1.

3.1. *Application of ZFN in genome editing of vector mosquitoes*

ZFN has been applied by a few researchers for the customized genome editing of vector mosquitoes. DeGennaro *et al*. targeted the *odorant receptor coreceptor* (*orco*) gene of *Aedes aegypti* to investigate the role of *orco* gene and the odorant receptor pathway in host identification and sensitivity to chemical repellent *N*,*N*-diethyl-meta-toluamide (DEET) (*14*). In this experiment, the designed ZFN was injected into *Ae. aegypti* embryos. The *orco* mutants generated through this study showed reduced spontaneous activity and reduced odour-evoked responses when compared to wild type. Behaviorally, *orco* mutant mosquitoes did not respond to human scent in the absence of CO_2. In another study, the ZFN was used to generate *neuropeptide Y-like receptors 1* (*npylr1*) null mutants to study the functional genomics. ZFN construct was injected into *Ae. aegypti* embryos at a concentration of 200 ng/μL and a homologous recombination vector at 850 ng/μL. The tested *npylr1* mutants did not inhibit the host-seeking behavior and the study concluded that other peptides may act with *npylr1* and regulate this host-seeking behavior (*45*). McMeniman *et al*. mutated the *Ae. aegypti gustatory receptors* (*AaegGr3*) gene, a subunit of the heteromeric CO_2 receptor by injecting ZFNs into pre-blastoderm stage embryos and reported that *Gr3* mutant of *Ae. aegypti* lacked electrophysiological and behavioral responses to CO_2 (*46*). These studies have confirmed that in spite of its complexity, ZFN could be successfully used for generating knock-out mutants in a major vector *Ae. aegypti*. May be the foundation laid with these studies could be utilized for further studies to advance the genome based control of mosquito-borne diseases using more convenient genome editing tools like CRISPR/Cas9.

3.2. *Application of TALEN in genome editing of vector mosquitoes*

In addition to ZFN mentioned above, TALEN has also been used as a potent genome editing tool to mutate the targeted genes in disease causing mosquitoes. Aryan *et al*. designed the TALEN to target the *kynurenine 3-monoxygenase* (*kmo*) gene of *Ae. aegypti* whose protein product was essential for the production of eye pigmentation (*13*). They injected the *kmo*-targeting TALEN construct into pre-blastoderm embryos of the black-eyed *Ae. aegypti*. Their assay resulted in 20-40% fertile survivors and most of them produced more than 20% white eyed progeny, with some producing up to 75% eye pigmentation mutants. Further, a detailed procedure for target selection (*kmo* gene), assessing the activity of TALEN, embryonic microinjection and detection of target site mutations in *Ae. aegypti* genome was described by the same group in the following year (*47*). In another reverse genetics study, Smidler *et al*. reported the targeted disruption of the *thioester-containing protein1* (*TEP1*) gene using TALEN in *Anopheles gambiae* mosquitoes which spreads malaria. *TEP1* is reported to be an immunity gene in *An. gambiae* against plasmodium infection (*15*). The induced mutations showed reduced protein production and the resulted *TEP1* mutants were hyper-susceptible to *Plasmodium berghei* infections. These studies have demonstrated the TALEN based genome alterations

in vector mosquitoes. As the designing of TALEN is easier than ZFN, the former may be utilized in future for customized editing of more potent genes in mosquito to control the spread of vector-borne diseases.

3.3. *Application of CRISPR/Cas9 in genome editing of vector mosquitoes*

As CRIPSR/Cas9 has emerged as a most popular and user friendly genome editing tool, it has opened new avenues for the editing of mosquito genomes with little effort. Several labs have already attempted this technique to engineer the genome of vector mosquitoes. Kistler *et al*. investigated the efficiency of CRISPR/Cas9-mediated gene editing system with *Aaeg-wtrw* locus to generate mutations via disparate repair mechanisms and achieved different types of mutations in several genomic loci of *Ae. aegypti* mosquitoes (*48*). Multiplexed effect of CRISPR/Cas9 was utilized by Basu *et al*. targeting 6 different (*kmo*, *loqs*, *r2d2*, *ku70*, *lig4* and *nix*) genes using CRISPR/Cas9 tool in *Ae. aegypti* mosquito (*49*). They considered that editing rate may vary across the genome. Hence they designed 40 additional sgRNAs and evaluated their editing potential in transient embryo assays and achieved generating different types of somatic and germline mutations in *Ae. aegypti* mosquitoes. These reports opened a new avenue for mosquito genome editing utilizing CRISPR/Cas9 system. In another study, Dong *et al*. used the CRISPR/Cas9-mediated system to modify *enhanced cyan fluorescent protein* (*ECFP*) gene in *Ae. aegypti* mosquito line expressing two different eye markers (*50*). Along with Cas9, two sgRNAs were used to target different regions of *ECFP* gene with *in vitro* transcribed mRNAs for germline transformation and obtained four different G1 pools with 5.5% knockout efficiency. The PCR amplification, cloning and sequencing experiments revealed indels (insertion or deletion) in the *ECFP* target gene ranging from 2-27 nucleotides and their results demonstrated that CRISPR/Cas9-mediated gene editing could be achievable in *Ae. aegypti* (*50*).

Another report in the same year by Hall *et al*. demonstrated that knockout of male determining (*Nix*) gene has resulted in feminized genetic males with successful application of CRISPR/Cas9-mediated gene editing system (*51*). Further, their investigation on ectopic expression of *Nix* gene in genetic females confirmed that *Nix* is sufficient to initiate male development and thus has given a path to convert the female mosquitoes into harmless male (*51*). This study may offer the possibilities of utilizing CRISPR/Cas9 for the customized editing of vector carrying female mosquito genomes. Studies like this could definitely lead to more meaningful inventions that will help combat the spread of deadly diseases especially in less developed countries. A recent review by Adelman and Tu also emphasized the importance of exploiting *Nix* gene for the control of mosquito borne infectious diseases (*31*).

Gantz *et al*. developed a CRISPR/Cas9-mediated gene editing system in the Asian malaria vector *An. stephensi* (*52*). This system produced progeny for a small number of generations that were derived from transgenic males exhibiting a high frequency of gene alteration that were consistent with homology-directed repair (HDR). It has been confirmed that CRISPR/Cas9 system copied a ~17-kb construct from its site of insertion to its homologous chromosome in a site-specific manner. The authors used dual anti-*Plasmodium falciparum* effector genes with a marker gene for this study and the gene-drive components were introgressed into ~99.5% of the progeny following outcrosses of transgenic lines to wild-type mosquitoes. This study provided evidence for a highly efficient gene-drive system that can spread anti-malarial genes such as *m1C3*, *m2A10* into the *An. stephensi* population (*52*). This could be utilized in future for efficient genome editing of *An. stephensi* which spreads malaria in less developed countries in Asia.

Hammond *et al*. targeted three female-fertility genes viz. *AGAP005958*, *AGAP007280*, *AGAP011377* of *An. gambiae* which were ortholog with *Drosophila* genes and made an attempt to disrupt the coding sequence of these genes using CRISPR/Cas9. They found that reproductive phenotypes (fertility) of the generated mutants suppressed mosquito population to levels that did not support malaria transmission. The role of these genes occurs at distinct stages of egg production and embryo development. The fertility assays in G_2 progeny showed that the homozygous mutant females were sterile, whereas heterozygous females showed normal rates of egg laying and larval emergence. Homozygous mutant females carrying disrupted genes either *AGAP005958* or *AGAP011377* failed to lay eggs, whereas *AGAP007280* gene disrupted homozygous females laid eggs that failed to hatch (*53*). Further, the team advanced their study and described the first functional CRISPR/Cas9 sex-distortion system ($CRISPR^{SD}$) in the malaria mosquito *An. gambiae*. They designed a germline transformation construct where Cas9 endonuclease coding sequence was placed in a spermatogenesis-specific β2 tubulin promoter and the $CRISPR^{SD}$ construct was enclosed in *piggyBac* transformation vector. Among four transgenic lines tested, all the lines showed a strong sex-ratio distortion, with a male bias progeny ranging from 86.1% to 94.8% of males and the hatching rates varied between 83.6% and 93.2% (*54*).

As the CRISPR/Cas9 has been popularized and considered as a go to technique for genome editing, we can expect many more studies with CRIPR/Cas9 to produce knock-out and knock-in mutants in vector mosquitoes. The production of mutant strains like nonpathogenic mosquitoes, host seeking disturbed mosquitoes, production of only male mosquitoes and production of wingless mosquitoes using CRISPR/Cas9 would be the near future approaches that might

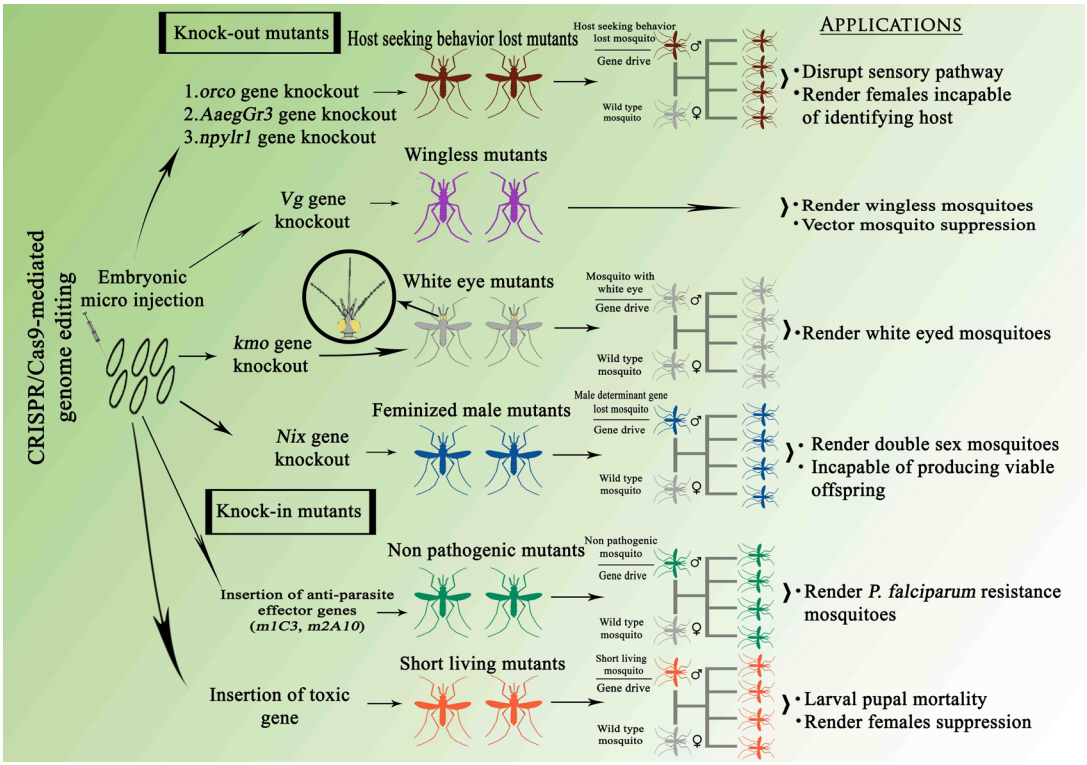

Figure 1. CRISPR-Cas9-mediated genetic modifications in mosquitoes. The figure shows two kinds of mutants such as knock-out and knock-in mutants produced by CRISPR/Cas9-mediated genome editing. It could be crossed with a wild type mosquito in a gene drive system to disrupt the particular activity/functions of the vector mosquito and population suppression that leads to disease eradication. The altered genes could be preferentially inherited by all offsprings when crossed with a wild type mosquito. A Gene drive technology would quickly spread the altered gene in the target mosquito population with nearly 100% chance. *Vg* - vestigial gene.

control/eradicate the spread of deadly diseases by vector mosquito species (Figure 1). Multiplexing feature of this tool also offers more possibilities to study the genes involved in mosquito-parasite interactions.

4. Off-target effects

Off-target mutations and unnecessary chromosomal translocations are the biggest issues with the genome editing tools. Especially with CRISPR/Cas9, specificity of sgRNA caused by off-target binding site mutations and co-inheritance caused by off-target mutations still need to be addressed to improve the specificity of genome editing tools for the successful application in mosquito control programmes (*55,56*). Furthermore, the off-target impacts of phylogenetic similarity, biogeographic overlap, and ecology, ecological resemblance with other non-target organisms and behavior of the mutants should be addressed in controlled small scale field trials (*46*).

Even though CRISPR/Cas9 is cost effective, the initial versions of CRISPR/Cas9 system had key issues due to off-target effects (*57,58*). Cas9 nickase (Cas9D10A mutant) was capable of creating single strand nicks (*59,60*), which when paired by targeting a site with two sgRNAs, resulted in a DSB which helped to overcome off target effects. More recently, Kleinstiver *et al*. developed a high-fidelity version of the Cas9 from *Streptococcus pyogenes* (SpCas9-HF1) which was an engineered variant of wild type Cas9 to reduce non-specific DNA contacts. This novel enzyme has greatly reduced the off-target effects in human cells when tested (*61*). This enzyme could be employed in genome editing of vector mosquitoes too in the near future to produce more specific gene alterations. Unlike ZFN and TALEN, software tools are available to predict the off-target effects of CRISPR/Cas9 system for each experiment. A software tool in the name of Digenome-seq was developed by South Korean scientists recently (*62*). This *in vitro* digest yielded sequence reads with the same 5′ ends at cleavage sites that could be computationally identified. The group had validated off-target sites at which mutations were induced with frequencies below 0.1%, near the detection limit of targeted deep sequencing. These recent developments on tools to predict the off-target effects will be helpful to avoid any undesirable effect of genome editing.

5. Ethical concerns

A great breakthrough was seen in UK recently in the era of genome editing. Developmental biologist Kathy Niakan of Francis Crick Institute in London has received permission from UK authorities to modify human embryos using the CRISPR/Cas9 gene-editing technology. Niakan applied for permission to use the technique to better understand the role of key genes

during the first few days of human embryo development (*63*). This has affirmed the promising results offered by CRISPR/Cas9 within a short period of its introduction. However it is really difficult to escape from critics and the protesters of genome editing. Although the genome editing is proved to be effective and useful, the editing rates may vary across the genome and also depend on the type of tool used. Each method has various disadvantages in terms of cost, sequence-specificity and off-target effects (*64,65*). Hence, genome editing raises many ethical issues and concerns to humans, other organisms and environment. Altering a gene in vector mosquitoes and releasing it in the environment could result in unknown and undesirable outcomes in the ecosystem. Many insect ecologists are deeply worried about the risk of mutated organisms and emergence of new insect pest. Ledford has also predicted the consequences and unpredictable effects of genome editing. Thus, the spread of genome edited strains through wild populations, would be extremely difficult to detect and would be challenging to biosecurity measures to prevent the spread of mutated mosquitoes, if they create unwanted effects (*66*). Similarly, the targeted removal of vector mosquito populations or spreading a genetic element to wild mosquito populations with CRISPR/Cas9-mediated gene drive technology may result in indirect ecological consequences and may raise various societal and regulatory concerns (*67,68*). Other concerns like survival rate of edited mosquitoes in the natural environment, effects on predatory insects and fishes who eat genetically edited mosquito larvae and ecological imbalance caused by vector mosquito population and eradication are still under debate. It should be carefully analyzed to produce only positive outcomes in the ecosystem.

6. The current problems in application of genome editing techniques in vector mosquitoes

Although user friendly genome editing tool CRISPR/Cas9 has enabled more rapid and efficient editing of mosquito genome with little effort, it has also caused some problems with off-target effects. Several studies have reported that Cas9 is prone to cutting off-target sequences that are similar to the target (*58,69-71*). Any such off-target effect may cause serious problems in vector mosquito genome editing. Mosquitoes such as *Ae. aegypti* have a very large genome of 1.38 Gb which may require more precise target site selection due to the increased number of potential off-target sequences present. Although many software tools are available to predict the off-target effects (reviewed in *43*), it was not possible to apply these tools for all mosquito species due to lack of complete genome in many species. Further, highly efficient mutation systems need to be explored; in many studies, only half of the treated populations receive the desired changes (success rate varies).

There is a serious problem that exists in the gene drive strategy to eradicate or suppress the vector mosquito populations using HDR. We have to ensure that the cut sequence should be repaired using HDR rather than NHEJ to copy the drive, to have a successful gene drive. Also, the gene drives should be activated only in germline cells and only at developmental stages with a high rate of HDR, but this may be challenging in some species of vector mosquitoes.

There is also an appropriate concern that spread of the gene drive in vector mosquitoes will be difficult to control, and may result in unwanted consequences beyond the expected level (*56,72,73*). Releasing genome edited strain in the environment could result in undesirable effects in the ecosystem. Further, there is no method available so far to detect the mutated mosquitoes in the field condition (*66*). Even a very efficient endonuclease gene drive may be vulnerable to the evolution of drive resistance in the natural population. If a cut is repaired using the NHEJ pathway instead of HDR, by error, the result will be typically a drive-resistant allele which will bring about undesirable effects. It has also been predicted that some natural sequence polymorphisms in the mosquito population may also prevent the precise cutting. Further, the gene drives require many generations to spread through populations to eliminate or suppress the population of mosquitoes.

In practice, after designing the gene drive in transgenic mosquitoes, they must be allowed to mate with wild-type individuals in order to begin the process of spreading the drive through the wild population. Several critical factors are involved in the successful spread of gene drive in the ecosystem with wild populations. The total time required to spread to all wild mosquitoes depends on several important factors including the number of drive carrying mosquitoes released into the ecosystem and efficiency of gene drive.

7. Future directions

Genome editing tools have been shown to have a great impact on vector mosquito genome modification and these tools can potentially be used further to study the functions of target gene, gene indels, inversions, duplications, genetic network system, polymorphisms and also to investigate the mosquito-pathogen gene interactions. In particular, CRISPR/Cas9-mediated gene editing system may emerge as an efficient tool and may occupy a predominant position with high frequency of target specificity to modify genes of vector mosquitoes. We also need to consider that the CRISPR/Cas9-based introduction of mosquitoes with modified gene may lead to alteration in the wild mosquito population and can result in extinction of the target mosquito species within a short period. However, it is more important to follow all the guidelines and strict biosafety measures to prevent unexpected and undesirable outcome of genome editing.

According to Webber *et al*. researchers, resource managers and policymakers must carefully weigh the risks of implementation of genome editing technologies like CRISPR/Cas9 that could threaten rather than assist a given ecosystem. For example CRISPR/Cas9 approach can be used as a "silver bullet" to manage highly threatening invasive alien species. They also suggest that there are several important factors to take into account especially compared with classical biological control methods which offer important insights in this context (*74*). The genome editing technology especially like CRISPR/Cas9 which has been invented recently and being much popularized has already started raising debates among the policy makers, governments, non-governmental organizations (NGOs) and public, like GM era in the past. However scientists around the world have the opportunity to make the best use of it to improve the process and product in their field of research for the betterment of humankind especially in the control of vector mosquitoes.

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References

1. Reegan AD, Gandhi MR, Paulraj MG, Balakrishna K, Ignacimuthu S. Effect of niloticin, a protolimonoid isolated from *Limonia acidissima* L.(Rutaceae) on the immature stages of dengue vector *Aedes aegypti* L.(Diptera: Culicidae). Acta Trop. 2014; 139:67-76.
2. WHO. World malaria report. World Health Organization. *http://www.who.int/malaria /publications/world_malaria_report_2014/report/en/ 2014* (accessed September 22, 2016).
3. WHO. WHO Director-General summarizes the outcome of the Emergency Committee regarding clusters of microcephaly and Guillain-Barré syndrome. *http://www.who.int/mediacentre/news/statements/2016/emergency-committee-zika-microcephaly/en/ 2016* (accessed September 22, 2016).
4. Bayen S. Occurrence, bioavailability and toxic effects of trace metals and organic contaminants in mangrove ecosystems: A review. Environ Int. 2012; 48:84-101.
5. Madhu SK, Shaukath AK, Vijayan VA. Efficacy of bioactive compounds from *Curcuma aromatica* against mosquito larvae. Acta Trop. 2010; 113:7-11.
6. Tikar S, Kumar A, Prasad G, Prakash S. Temephos-induced resistance in *Aedes aegypti* and its cross-resistance studies to certain insecticides from India. Parasitol Res. 2009; 105:57-63.
7. Catteruccia F, Nolan T, Loukeris TG, Blass C, Savakis C, Kafatos FC, Crisanti A. Stable germline transformation of the malaria mosquito *Anopheles stephensi*. Nature. 2000; 405:959-962.
8. Coates CJ, Jasinskiene N, Miyashiro L, James AA. Mariner transposition and transformation of the yellow fever mosquito, *Aedes aegypti*. Proc Natl Acad Sci. 1998; 95:3748-3751.
9. Grossman GL, Rafferty CS, Clayton JR, Stevens TK, Mukabayire O, Benedict MQ. Germline transformation of the malaria vector, *Anopheles gambiae*, with the piggyBac transposable element. Insect Mol Biol. 2001; 10:597-604.
10. Jasinskiene N, Coates CJ, Benedict MQ, Cornel AJ, Rafferty CS, James AA, Collins FH. Stable transformation of the yellow fever mosquito, *Aedes aegypti*, with the Hermes element from the housefly. Proc Natl Acad Sci. 1998; 95:3743-3747.
11. Gabrieli P, Smidler A, Catteruccia F. Engineering the control of mosquito-borne infectious diseases. Genome Biol. 2014; 15:1-9.
12. Knipling E. Possibilities of insect control or eradication through the use of sexually sterile males. J Econ Entomol. 1955; 48:459-462.
13. Aryan A, Anderson MAE, Myles KM, Adelman ZN. TALEN-based gene disruption in the dengue vector *Aedes aegypti*. Plos One. 2013; 8:e60082.
14. DeGennaro M, McBride CS, Seeholzer L, Nakagawa T, Dennis EJ, Goldman C, Jasinskiene N, James AA, Vosshall LB. *orco* mutant mosquitoes lose strong preference for humans and are not repelled by volatile DEET. Nature. 2013; 498:487-491.
15. Smidler AL, Terenzi O, Soichot J, Levashina EA, Marois E. Targeted mutagenesis in the malaria mosquito using TALE nucleases. PLoS One. 2013; 8:e74511.
16. Criscione F, O'Brochta DA, Reid W. Genetic technologies for disease vectors. Curr Opin Insect Sci. 2015; 10:90-97.
17. Bibikova M, Beumer K, Trautman JK, Carroll D. Enhancing gene targeting with designed zinc finger nucleases. Science. 2003; 300:764-764.
18. Bibikova M, Golic M, Golic KG, Carroll D. Targeted chromosomal cleavage and mutagenesis in *Drosophila* using zinc-finger nucleases. Genetics. 2002; 161:1169-1175.
19. Boch J, Bonas U. Xanthomonas AvrBs3 family-type III effectors: Discovery and function. Annu Rev Phytopathol. 2010; 48:419-436.
20. Curtin SJ, Zhang F, Sander JD, Haun WJ, Starker C, Baltes NJ, Reyon D, Dahlborg EJ, Goodwin MJ, Coffman AP, Dobbs D, Joung JK, Voytas DF, Stupar RM. Targeted mutagenesis of duplicated genes in soybean with zinc-finger nucleases. Plant Physiol. 2011; 156:466-473.
21. Sander JD, Dahlborg EJ, Goodwin MJ, Cade L, Zhang F, Cifuentes D, Curtin SJ, Blackburn JS, Thibodeau-Beganny S, Qi Y, Pierick CJ, Hoffman E, Maeder ML, Khayter C, Reyon D, Dobbs D, Langenau DM, Stupar RM, Giraldez AJ, Voytas DF, Peterson RT, Yeh JR, Joung JK. Selection-free zinc-finger-nuclease engineering by context-dependent assembly (CoDA). Nat Methods. 2011; 8:67-69.
22. Shukla VK, Doyon Y, Miller JC, DeKelver RC, Moehle EA, Worden SE, Mitchell JC, Arnold NL, Gopalan S, Meng X, Choi VM, Rock JM, Wu YY, Katibah GE, Zhifang G, McCaskill D, Simpson MA, Blakeslee B, Greenwalt SA, Butler HJ, Hinkley SJ, Zhang L, Rebar EJ, Gregory PD, Urnov FD. Precise genome

modification in the crop species Zea mays using zinc-finger nucleases. Nature. 2009; 459:437-441.
23. Carlson DF, Tan W, Lillico SG, Stverakova D, Proudfoot C, Christian M, Voytas DF, Long CR, Whitelaw CBA, Fahrenkrug SC. Efficient TALEN-mediated gene knockout in livestock. Proc Natl Acad Sci. 2012; 109:17382-17387.
24. Cermak T, Doyle EL, Christian M, Wang L, Zhang Y, Schmidt C, Baller JA, Somia NV, Bogdanove AJ, Voytas DF. Efficient design and assembly of custom TALEN and other TAL effector-based constructs for DNA targeting. Nucleic Acids Res. 2011; 39:e82.
25. Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science. 2012; 337:816-821.
26. Carroll D. Genome Engineering with Zinc-Finger Nucleases. Genetics. 2011; 188:773-782.
27. Callaway E. Mosquitoes engineered to pass down genes that would wipe out their species. Nature | News 2015.
28. Ledford H, Callaway E. Gene drive' mosquitoes engineered to fight malaria. Nature | News. 2015.
29. Overcash JM, Aryan A, Myles KM, Adelman ZN. Understanding the DNA damage response in order to achieve desired gene editing outcomes in mosquitoes. Chromosome Res. 2015; 23:31-42.
30. Champer J, Buchman A, Akbari OS. Cheating evolution: Engineering gene drives to manipulate the fate of wild populations. Nat Rev Genet. 2016; 17:146-159.
31. Adelman ZN, Tu Z. Control of mosquito-borne infectious diseases: Sex and gene drive. Trends Parasitol. 2016; 32:219-229.
32. Chakradhar S. Buzzkill: Regulatory uncertainty plagues rollout of genetically modified mosquitoes. Nat Med. 2016; 21:416-418.
33. Bibikova M, Carroll D, Segal DJ, Trautman JK, Smith J, Kim YG, Chandrasegaran S. Stimulation of homologous recombination through targeted cleavage by chimeric nucleases. Mol Cell Biol. 2001; 21:289-297.
34. Kim YG, Cha J, Chandrasegaran S. Hybrid restriction enzymes: Zinc finger fusions to Fok I cleavage domain. Proc Natl Acad Sci. 1996; 93:1156-1160.
35. Miller J, McLachlan AD, Klug A. Repetitive zinc-binding domains in the protein transcription factor IIIA from *Xenopus* oocytes. EMBO J. 1985; 4:1609-1614.
36. Wolfe SA, Nekludova L, Pabo CO. DNA recognition by Cys2His2 zinc finger proteins. Annu Rev Biophy Biomol Struct. 2000; 29:183-212.
37. Reyon D, Tsai SQ, Khayter C, Foden JA, Sander JD, Joung JK. FLASH assembly of TALENs for high-throughput genome editing. Nat Biotechnol. 2012; 30:460-465.
38. Mussolino C, Cathomen, T. TALE nucleases: Tailored genome engineering made easy. Curr Opin Biotechnol. 2012; 23:644-650.
39. Sakuma T, Woltjen K. Nuclease-mediated genome editing: At the front-line of functional genomics technology. Dev Growth Differ. 2014; 56:2-13.
40. Grissa I, Vergnaud G, Pourcel C. The CRISPRdb database and tools to display CRISPRs and to generate dictionaries of spacers and repeats. BMC Bioinformatics. 2007; 8:1-10.
41. Doudna JA, Charpentier E. The new frontier of genome engineering with CRISPR-Cas9. Science. 2014; 346):1-9.
42. Barrangou R, Dounda JA. Applications of CRISPR technologies in research and beyond. Nat Biotech. 2016; 34:933-941.
43. Ceasar A, Rajan V, Prykhozhij SV, Berman JN, Ignacimuthu S. Insert, remove or replace: A highly advanced genome editing system using CRISP/Cas9 BBA Mol Cell Res. 2016; 1863:2333-2344.
44. Franz AW, Clem RJ, Passarelli AL. Novel genetic and molecular tools for the investigation and control of dengue virus transmission by mosquitoes. Curr Trop Med Rep. 2014; 1:21-31.
45. Liesch J, Bellani LL, Vosshall LB. Functional and genetic characterization of neuropeptide Y-like receptors in *Aedes aegypti*. PLoS Negl Trop Dis. 2013; 7:e2486.
46. McMeniman CJ, Corfas RA, Matthews BJ, Ritchie SA, Vosshall LB. Multimodal integration of carbon dioxide and other sensory cues drives mosquito attraction to humans. Cell. 2014; 156:1060-1071.
47. Aryan A, Myles KM, Adelman ZN. Targeted genome editing in *Aedes aegypti* using TALENs. Methods. 2014; 69:38-45.
48. Kistler KE, Vosshall LB, Matthews BJ. Genome engineering with CRISPR-Cas9 in the mosquito *Aedes aegypti*. Cell Reports. 2015; 11:51-60.
49. Basu S, Aryan A, Overcash JM, Samuel GH, Anderson MAE, Dahlem TJ, Myles KM, Adelman ZN. Silencing of end-joining repair for efficient site-specific gene insertion after TALEN/CRISPR mutagenesis in *Aedes aegypti*. Proc Natl Acad Sci. 2015; 112:4038-4043.
50. Dong S, Lin J, Held NL, Clem RJ, Passarelli AL, Franz AW. Heritable CRISPR/Cas9-mediated genome editing in the yellow fever mosquito, *Aedes aegypti*. Plos One. 2015; 10:e0122353.
51. Hall AB, Basu S, Jiang X, Qi Y, Timoshevskiy VA, Biedler JK, Sharakhova MV, Elahi R, Anderson MA, Chen XG, Sharakhov IV, Adelman ZN, Tu Z. A male-determining factor in the mosquito *Aedes aegypti*. Science. 2015; 348:1268-1270.
52. Gantz VM, Jasinskiene N, Tatarenkova O, Fazekas A, Macias VM, Bier E, James AA. Highly efficient Cas9-mediated gene drive for population modification of the malaria vector mosquito *Anopheles stephensi*. Proc Natl Acad Sci. 2015; 112:E6736-E6743.
53. Hammond A, Galizi R, Kyrou K, Simoni A, Siniscalchi C, Katsanos D, Gribble M, Baker D, Marois E, Russell S, Burt A, Windbichler N, Crisanti A, Nolan T. A CRISPR-Cas9 gene drive system targeting female reproduction in the malaria mosquito vector *Anopheles gambiae*. Nat Biotechnol. 2016; 34:78-83.
54. Galizi R, Hammond A, Kyrou K, Taxiarchi C, Bernardini F, O'Loughlin SM, Papathanos PA, Nolan T, Windbichler N, Crisanti A. A CRISPR-Cas9 sex-ratio distortion system for genetic control. Scientific Reports. 2016; 6:1-5.
55. Briese DT. Weed biological control: Applying science to solve seemingly intractable problems. Aust J Entomol. 2004; 43:304-317.
56. Esvelt KM, Smidler AL, Catteruccia F, Church GM. Concerning RNA-guided gene drives for the alteration of wild populations. Elife. 2014; 3:e03401.
57. Cradick TJ, Fine EJ, Antico CJ, Bao G. CRISPR/Cas9 systems targeting β-globin and CCR5 genes have substantial off-target activity. Nucleic Acids Res. 2013; 41:9584-9592.
58. Fu Y, Foden JA, Khayter C, Maeder ML, Reyon D, Joung JK, Sander JD. High-frequency off-target

mutagenesis induced by CRISPR-Cas nucleases in human cells. Nat Biotechnol. 2013; 31:822-826.
59. Mali P, Yang L, Esvelt KM, Aach J, Guell M, DiCarlo JE, Norville JE, Church GM. RNA-guided human genome engineering via Cas9. Science. 2013a; 339:823-826.
60. Ran FA, Hsu PD, Lin CY, Gootenberg JS, Konermann S, Trevino AE, Scott DA, Inoue A, Matoba S, Zhang Y, Zhang F. Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. Cell. 2013; 154:1380-1389.
61. Kleinstiver BP, Pattanayak V, Prew MS, Tsai SQ, Nguyen NT, Zheng Z, Joung JK. High-fidelity CRISPR-Cas9 nucleases with no detectable genome-wide off-target effects. Nature. 2016; 529:490-495.
62. Kim D, Bae S, Park J, Kim E, Kim S, Yu HR, Hwang J, Kim JI, Kim JS. Digenome-seq: Genome-wide profiling of CRISPR-Cas9 off-target effects in human cells. Nat Methods. 2015; 12:237-243.
63. Vogel G. U.K. researcher receives permission to edit genes in human embryos. Science News. *http://www.sciencemag.org/news/2016/02/uk-researcher-receives-permission-edit-genes-human-embryos* (accessed September 22, 2016).
64. DeFrancesco L. Move over ZFNs. Nature Biotechnol. 2012; 29:681-684.
65. Gaj T, Gersbach CA, Barbas CF. ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. Trends Biotechnol. 2013; 31:397-405.
66. Ledford H. CRISPR, the disruptor. Nature. 2015; 522:20-24.
67. Alphey L. Can CRISPR-Cas9 gene drives curb malaria? Nat Biotechnol. 2016; 34:149-150.
68. Paulraj MG, Ignacimuthu S, Reegan AD. Gene silencing and gene drive in dengue vector control: A review. Indian J. Nat Prod Res. 2016; 7:1-8.
69. Hsu PD, Scott DA, Weinstein JA, Ran FA, Konermann S, Agarwala V, Li Y, Fine EJ, Wu X, Shalem O, Cradick TJ, Marraffini LA, Bao G, Zhang F. DNA targeting specificity of RNA-guided Cas9 nucleases. Nat Biotechnol. 2013; 31:827-832.
70. Mali P, Aach J, Stranges PB, Esvelt KM, Moosburner M, Kosuri S, Yang L, Church GM. CAS9 transcriptional activators for target specificity screening and paired nickases for cooperative genome engineering. Nat Biotechnol. 2013b; 31:833-838.
71. Pattanayak V, Lin S, Guilinger JP, Ma E, Doudna JA, Liu DR. High-throughput profiling of off-target DNA cleavage reveals RNA-programmed Cas9 nuclease specificity. Nat Biotechnol. 2013; 31:839-843.
72. Burt A. Site-specific selfish genes as tools for the control and genetic engineering of natural populations. Proc Biol Sci. 2003; 270:921-928.
73. Oye KA, Esvelt K, Appleton E, Catteruccia F, Church G, Kuiken T, Lightfoot SB, McNamara J, Smidler A, Collins JP. Regulating gene drives. Science. 2014; 345:626-628.
74. Webber BL, Raghu S, Edwards OR. Opinion: Is CRISPR-based gene drive a biocontrol silver bullet or global conservation threat? Proc Natl Acad Sci. 2015; 112:10565-10567.

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